

A mouse model of familial porphyria cutanea tarda

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Approximately one-third of patients with porphyria cutanea tarda (PCT), the most common porphyria in humans, inherit a single mutant allele of the uroporphyrinogen decarboxylase (*URO-D*) gene. PCT associated with *URO-D* mutations is designated familial PCT. The phenotype is characterized by a photosensitive dermatosis with hepatic accumulation and urinary excretion of uroporphyrin and hepta-carboxylic porphyrins. Most heterozygotes for *URO-D* mutations do not express a porphyric phenotype unless hepatic siderosis is present. Hemochromatosis gene (*HFE*) mutations are frequently found when the phenotype is expressed. We used homologous recombination to disrupt one allele of murine *URO-D*. *URO-D*^{+/-} mice had half-wild type (wt) *URO-D* protein and enzymatic activity in all tissues but did not accumulate hepatic porphyrins, indicating that half-normal *URO-D* activity is not rate limiting. When *URO-D*^{+/-} mice were injected with iron-dextran and given drinking water containing δ -aminolevulinic acid for 21 days, hepatic porphyrins accumulated, and hepatic *URO-D* activity was reduced to 20% of wt. We bred mice homozygous for an *HFE* gene disruption (*HFE*^{-/-}) to *URO-D*^{+/-} mice, generating mice with the *URO-D*^{+/-}/*HFE*^{-/-} genotype. These animals developed a porphyric phenotype by 14 weeks of age without ALA supplementation, and *URO-D* activity was reduced to 14% of wt. These data indicate that iron overload alone is sufficient to reduce *URO-D* activity to rate-limiting levels in *URO-D*^{+/-} mice. The *URO-D*^{+/-} mouse serves as an excellent model of familial PCT and affords the opportunity to define the mechanism by which iron influences *URO-D* activity.

Porphyria cutanea tarda (PCT), the most common porphyric disorder in humans, is estimated to occur with a prevalence of one in 5,000 (1). Clinically, PCT is characterized by cutaneous photosensitivity associated with skin fragility and blistering. Biochemically, the disorder is characterized by the accumulation of uroporphyrin and hepta-carboxyl porphyrin in the liver. These compounds circulate in plasma, mediate the cutaneous photosensitivity, and are excreted in the urine. Diminished activity of uroporphyrinogen decarboxylase (*URO-D*) in the liver is present in all cases, and hepatic iron overload is a nearly constant finding (2, 3).

Heterozygosity for mutant *URO-D* alleles is present in approximately one-third of patients with PCT (1). The activity of *URO-D* and the concentration of *URO-D* protein are half-normal in all tissues, but porphyrins accumulate predominantly in the liver. This type of PCT is designated familial-PCT (F-PCT). Most carriers of mutant *URO-D* alleles do not express a porphyric phenotype unless hepatic siderosis develops, often in association with homozygosity for the cysteine 282 tyrosine (C282Y) mutation of the hemochromatosis gene (*HFE*; refs. 1, 4, and 5). Environmental factors such as alcohol abuse, hepatitis C virus, and estrogen use also play a role in clinical expression (1, 5). Numerous mutations of the *URO-D* gene have been identified in patients with F-PCT (6–15) but the most common is a splice-site mutation resulting in the deletion of exon 6 (16).

Approximately two-thirds of cases of PCT occur in individuals with wild-type (wt) *URO-D* alleles. This type of PCT has been designated sporadic-PCT. *URO-D* protein concentration and catalytic activity are normal in all tissues but in the liver; protein

levels are normal, and enzymatic activity is severely reduced. As with F-PCT, both environmental factors (alcohol, hepatitis C, and estrogens) and genetic factors (*HFE* mutations) play a role in clinical expression (1, 4, 5), and depletion of liver iron stores is central to successful therapy.

URO-D catalyzes the decarboxylation of the four acetate side chains of uroporphyrinogen to yield coproporphyrinogen (Fig. 1). *URO-D* is a homodimer with a molecular mass of 82 kDa (17) and is a member of the TIM barrel protein family, with one active site per monomer (18). The *URO-D* gene has been cloned and characterized from many species, and there is strong amino acid sequence homology among species (2, 18). A murine *URO-D* cDNA has been cloned (19), but the mouse *URO-D* genomic locus has not yet been characterized.

An outbreak of PCT occurred in Turkey in 1960 after seed wheat treated with the fungicide hexachlorobenzene was ingested rather than planted (20). Subsequently, many murine models of PCT induced by polyhalogenated aromatic compounds have been reported, but no animal models of F-PCT exist just as no *URO-D* mutations have been detected in mammals other than humans. To generate a mouse model of F-PCT, we cloned and characterized the *URO-D* gene from a mouse genomic library and used established gene-targeting techniques to generate mice that were heterozygous for a *URO-D*-null allele (*URO-D*^{+/-}). The *URO-D*^{+/-} mouse, like most humans heterozygous for *URO-D* mutations, did not develop a porphyric phenotype unless additional factors were present. Injection of iron-dextran coupled with drinking water supplemented with δ -aminolevulinic acid (ALA) produced a porphyric phenotype in 3 weeks. When *URO-D*^{+/-} animals were crossbred to *HFE*^{-/-} animals, a porphyric phenotype developed after 14 weeks without ALA supplementation or any other manipulation. Expression of the porphyric phenotype in both iron-injected *URO-D*^{+/-} mice and unperturbed *URO-D*^{+/-}/*HFE*^{-/-} animals was associated with reduction of hepatic *URO-D* activity to levels well below the half-wt value in untreated *URO-D*^{+/-} animals, even though *URO-D* protein levels remained constant. These findings strongly suggest that an inhibitor of *URO-D* was generated. This mouse model of F-PCT affords the opportunity to define the factors required to generate an inhibitor of *URO-D* activity without the need for any exogenous perturbing factors.

Materials and Methods

Cloning of *URO-D* from a Mouse Genomic Library. A total of 8.5×10^5 recombinants from a 129SvJ mouse genomic library (Strat-

Abbreviations: *URO-D*, uroporphyrinogen decarboxylase; *HFE*, hemochromatosis gene; PCT, porphyria cutanea tarda; F-PCT, familial PCT; ALA, δ -aminolevulinic acid; wt, wild-type; ES, embryonic stem.

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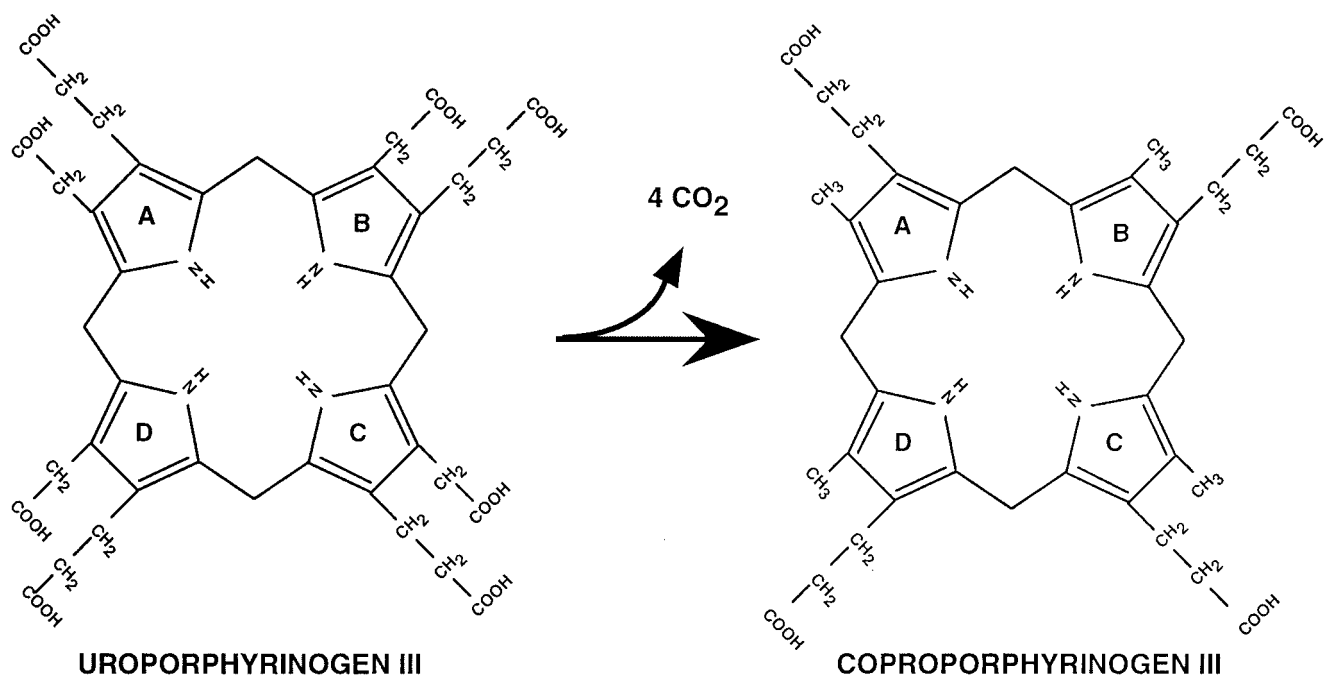


Fig. 1. Enzymatic conversion of uroporphyrinogen III to coproporphyrinogen III. At physiological substrate concentrations, the four acetate groups of uroporphyrinogen are decarboxylated in a clockwise fashion, starting with the acetate group on the asymmetric D ring (37). The enzyme will use any of the uroporphyrinogen isomers as substrates, but only the III isomer is used for heme production. Under conditions of substrate excess, decarboxylations occur in a random fashion (38). URO-D, a cytosolic 80-kDa homodimer, functions without the need for a cofactor (17, 18).

agene) were screened as described (21) with a radiolabeled full-length human *URO-D* cDNA (22). Positive plaques after the third screen were further confirmed by PCR using published primer pairs (23). Bacteriophage lambda DNA was prepared by purification of DNA from small-scale liquid cultures as described (24).

Targeted Disruption of Mouse *URO-D*. A DNA vector containing the disrupted allele of *URO-D* was introduced into murine embryonic stem (ES) cells by the method of Thomas and Capecchi (25). The linearized vector, pTKNEOURO-D (1.4 μ g), was transfected by electroporation into 1.4×10^7 R1 ES cells (26) suspended in 1.2 ml of transfer buffer (20 mM Hepes, pH 7.0/6 mM dextrose/137 mM NaCl/5 mM KCl/0.7 mM Na₂HPO₄/2 mM 2-mercaptoethanol). Immediately after electroporation, 2×10^6 ES cells were plated in 10 ml DMEM supplemented with 15% FCS onto eight 10-cm dishes, each seeded with 2.5×10^6 radiation-inactivated embryonic fibroblasts (27). After 24 h of growth, the media were replaced and supplemented with 280 μ g/ml neomycin (G418) and 2×10^6 M ganciclovir (28). After 7 days of growth, individual colonies were picked, and each was transferred to one well of a four-well culture plate (Nunc), also seeded with embryonic fibroblasts. When cells covered 80% of the well, they were removed by trypsinization and transferred to one well of a 12-well culture plate (Corning).

Screening of ES Cell Clones. The 140 ES cell clones, which were resistant to G418 and ganciclovir, were analyzed for integration of *NEO*^r at the correct genomic locus. Genomic DNA from each clone was purified (25) and digested with *Eco*RI, *Xba*I, or *Hind*III; electrophoresed through a 0.7% agarose gel; and transferred for Southern blot analysis. Membranes were probed with either a *Bam*HI to *Eco*RI fragment of the "F" fragment (Fig. 2A) or a DNA fragment containing the *NEO*^r gene. Mouse Cot-1 DNA (GIBCO/BRL) was added with the probe and

hybridized for 2.5 h at 65°C in Rapid-Hyb buffer (Amersham Life Science, Arlington Heights, IL) and washed in $0.1 \times$ SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) and 0.1% SDS. Blots were exposed to x-ray film (Kodak) for 24–72 h. Five clones with a correctly targeted integration were identified. The 1C3 clone was injected into C57BL/6J-derived blastocysts that were implanted into foster mothers who were allowed to come to term. Chimeric animals were identified by coat color, and males were mated with C57BL/6J mice to produce mice heterozygous at the *URO-D* locus.

URO-D Assays and Porphyrin Determinations. Activity of URO-D and tissue and urine porphyrin concentrations were determined as described (29). Porphyrin standards for HPLC determinations were purchased from Porphyrin Products (Logan, UT).

Iron Analysis in Liver Samples. Approximately 100 mg of liver tissue was added to 400 μ l of a nitric/perchloric acid mix [5:2 (vol/vol)] and incubated at 100°C for 4–6 h. Samples were centrifuged at $100,000 \times g$; the supernatant was diluted 1:2 (vol/vol) with water and analyzed on a Perkin-Elmer Optima 3100 XL instrument. Iron levels were normalized to original tissue wet weight.

PCR Genotyping at the *URO-D* Locus. Genotyping at the *URO-D* locus used DNA purified from tail biopsies or embryo sacs (30). The primers used to amplify wt *URO-D* (5' WF: CTG-GAGAAAGCAGGGATGTTACT with 3' primer R: CTGAG-GAAGGGCTAAGAATCTAC) amplify a 298-bp product. The mutant locus was amplified by using the 5' primer NF: CCT-GTTACCCTTTTGTTTGGATA and the common R, listed above, as the 3' primer, yielding a 196-bp product. Reactions contained 5 ng/ μ l genomic DNA in a standard PCR buffer mix as described by the manufacturer (Life Technologies, Rockville, MD; ref. 28). Amplification conditions were as follows: 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec for 30 cycles.

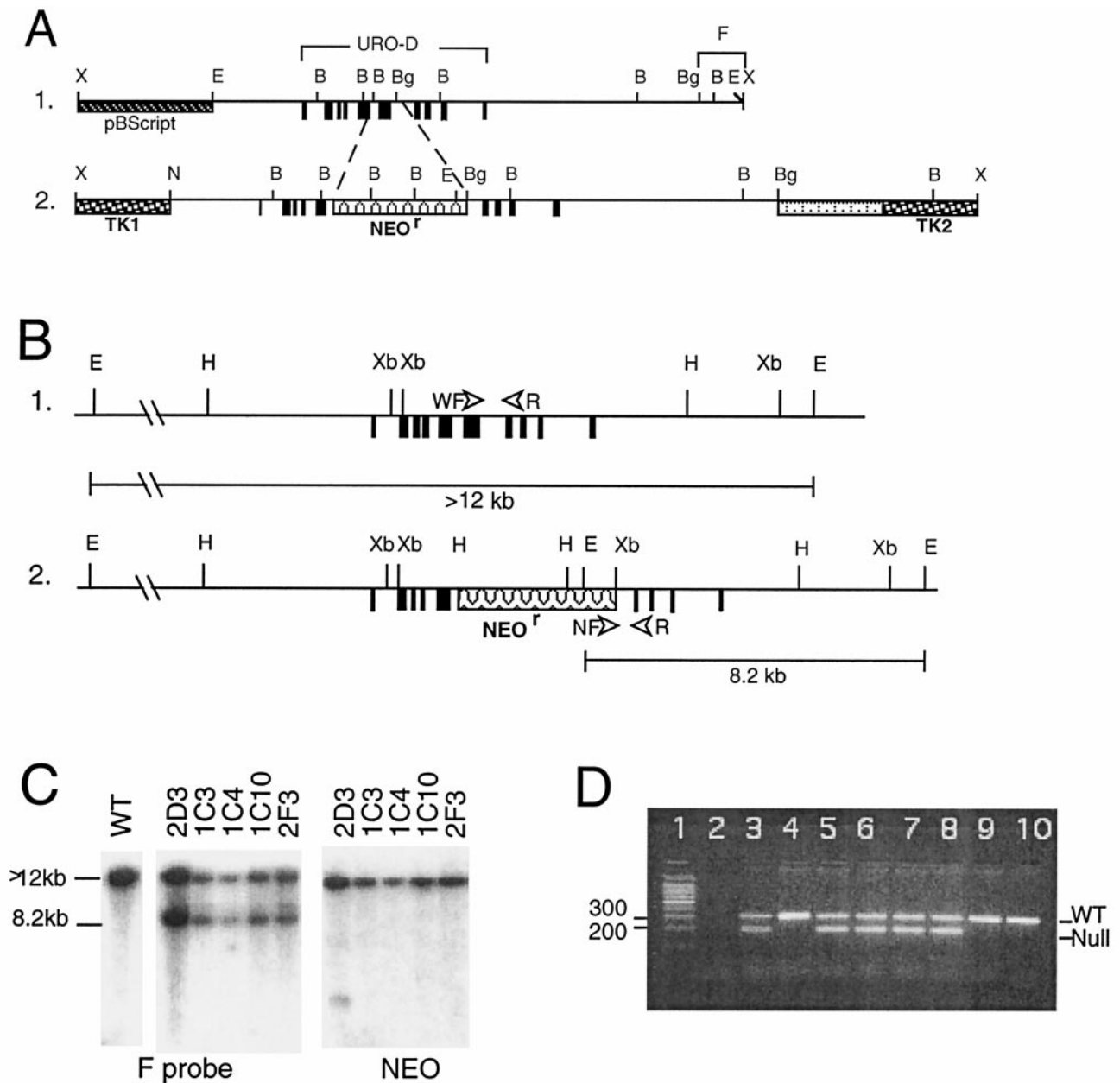


Fig. 2. Characterization of the null allele. (A1) A 12-kb *EcoRI* fragment from the genomic clone was subcloned into pBSIISK⁺. A 1-kb *Bgl*III to *Xho*I fragment (F) was removed. This fragment was subcloned and used as an external probe for Southern blot analysis. (A2) A fragment of DNA containing the neomycin resistance gene (*NEO^r*; ref. 27) was inserted between the *Bam*HI and *Bgl*III sites that flank exon 6, deleting exon 6 and portions of the flanking introns. The resulting genomic fragment was purified and ligated into the vector containing two copies of the herpes simplex virus thymidine kinase gene (27). The final construct pTKNEOURO-D was linearized with *Xho*I and transferred by electroporation into ES cells. (B) A restriction map of the wt allele (1) and the *NEO^r* disrupted allele (2) are shown. Approximate band size expected from digestion with *Eco*RI are shown as solid lines below each allele. Horizontal arrows represent primers (WF, wt forward; R, reverse; NF, *NEO* forward) used to generate PCR products shown in D. (C) Southern blot of wt DNA and DNA from ES cell lines carrying the targeted disruption. The DNA was digested to completion with *Eco*RI and probed with a *Bam*HI, *Eco*RI fragment of the F probe or a portion of the *NEO* gene. Molecular size markers are indicated on the right. (D) Genotyping by PCR. Genomic DNA was prepared from tails of mice and used for genotypic assignment (27). The three primers shown in B1 and B2 were used to amplify genomic DNA. The bands at 196 bp and 298 bp represent the disrupted (Null) and wt alleles, respectively. Restriction enzymes: *Bam*HI (B), *Bgl*III (Bg), *Eco*RI (E), *Hind*III (H), *Not*I (N), *Xho*I (X), and *Xba*I (Xb).

Hemochromatosis Gene (*HFE*) Knockouts. A generation of *HFE*^{-/-} mice was accomplished as described (31).

Results

Characterization of the Mouse *URO-D* Gene. The genomic architecture of the mouse *URO-D* gene proved to be identical to the human gene (22) with 10 exons and nine introns. The exons of murine *URO-D* were identical in length to the corresponding

exons in the human gene. The exonic sequence corresponded to the reported sequence of a mouse *URO-D* cDNA isolated from a B6/CBF1J mouse liver cDNA library (19) with two exceptions. We determined amino acid 96 to be methionine (encoded by ATG) rather than isoleucine and amino acid 333 to be tyrosine (encoded by TAC) rather than serine. The sequence we determined was consistent with the deduced amino acid sequences of human, rat, and yeast *URO-D* (22, 32, 33). Intron lengths were

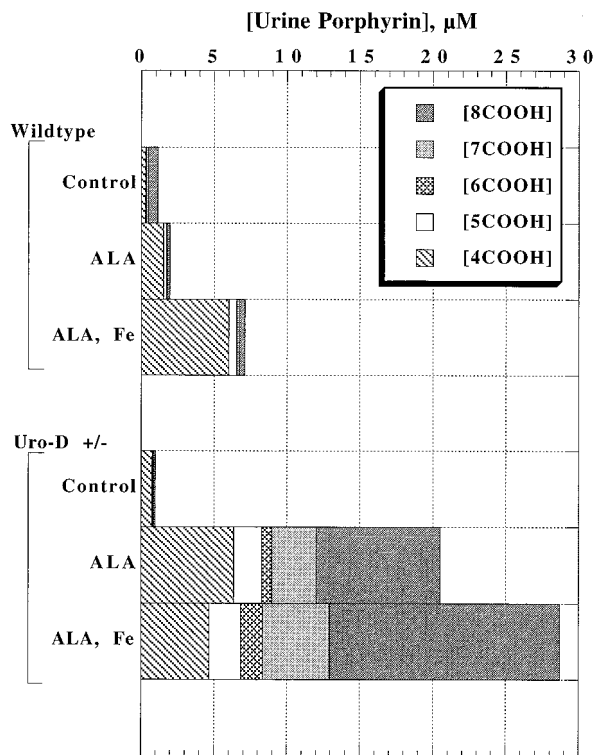


Fig. 3. Quantification of urinary porphyrins in wt and *URO-D*^{+/-} mice 3 weeks after treatment with ALA or ALA plus iron. Animals were given drinking water containing ALA (2 mg/ml), and some received an i.p. injection of iron-dextran (Fe) containing 10 mg of iron. Urinary porphyrins were separated and quantified by HPLC. Each group represents the mean value from three animals. Within a group, neither total urinary porphyrin excretion nor the proportion of uroporphyrin or partially decarboxylated porphyrins varied by more than 10%. 8COOH, uroporphyrin; 7COOH, heptacarboxyl porphyrin; 6COOH, hexacarboxyl porphyrin; 5COOH, pentacarboxyl porphyrin; 4COOH, coproporphyrin.

similar to the human gene with the exceptions of intron 6 (799 nt in mouse vs. 362 nt in human) and intron 9 (777 nt in mouse vs. 321 nt in human). We sequenced 600 bp of the 5' untranslated region and found the basic transcription elements of a house-keeping gene.

Disruption of the *URO-D* Gene in Mice. We used established gene-targeting techniques to delete exon 6 of the murine *URO-D* gene in ES cells (Fig. 2*A* and *B*; ref. 26). This deletion results in a null allele (Fig. 2*A*; ref. 16). The deletion was confirmed by Southern blotting (Fig. 2*C*). An ES cell line (1C3) containing the null allele was injected into blastocysts derived from C57BL/6J mice, and recombinant animals were generated and genotyped (Fig. 2*D*).

When *URO-D*^{+/-} animals were intercrossed, the ratio of the *URO-D*^{+/+}:*URO-D*^{+/-}:*URO-D*^{-/-} genotypes in offspring was 140:253:0 with approximately equal numbers of males and females of each genotype. To determine the gestational time of lethality for *URO-D*^{-/-} embryos, we examined 66 embryos between day 7.5 and 9.5 after fertilization; 20 were *URO-D*^{+/+}, 46 were *URO-D*^{+/-}, and none were *URO-D*^{-/-}, indicating that the *URO-D*^{-/-} genotype is an early embryonic lethal.

Exogenous Factors Are Required to Produce a Porphyrin Phenotype in *URO-D*^{+/-} Mice. The hepatic porphyrin content in *URO-D*^{+/-} mice did not differ from that in *URO-D*^{+/+} littermates, but *URO-D* protein quantified by Western immunoblotting and *URO-D* enzymatic activity was half-wt in liver, lung, kidney, and muscle (data not shown). Hepatic iron concentrations in *URO-D*^{+/-}

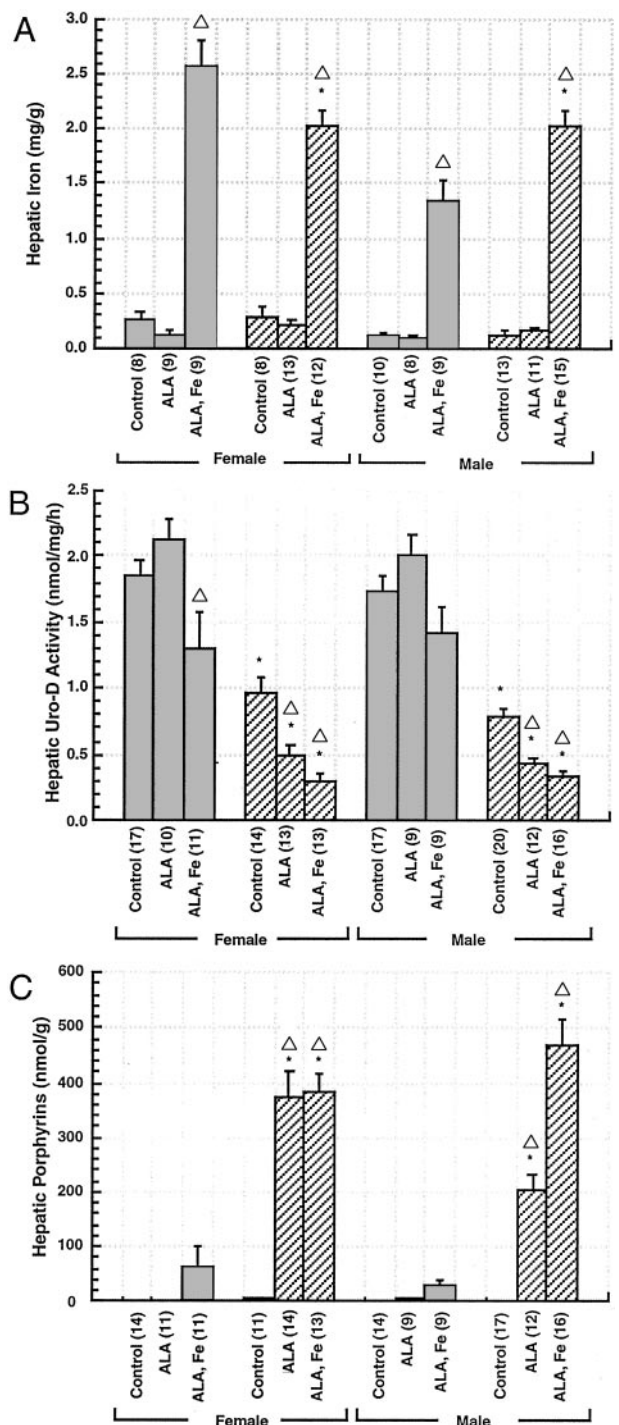


Fig. 4. Hepatic iron, *URO-D* activity, and porphyrin content in wt and *URO-D*^{+/-} mice 3 weeks after treatment with ALA or ALA plus iron. (A) Hepatic iron concentration, determined by atomic absorption spectroscopy. (B) Hepatic *URO-D* activity determined on cytosolic extracts. (C) Total hepatic porphyrin concentration. The numbers in parentheses in A–C represent the number of animals in each group. ■, wt; ▨, *URO-D*^{+/-}. * indicates a value significantly different from wt. Δ indicates a value significantly different from control animals in the same group. Error bars represent the standard error of the mean.

mice did not differ from *URO-D*^{+/+} animals followed up to 1 year of age, indicating that *URO-D* deficiency does not lead to hepatic iron accumulation.

Both *URO-D*^{+/+} and *URO-D*^{+/-} adult animals (age range 3 mo

Table 1. Effects of *URO-D* and *HFE* genotypes on hepatic iron and porphyrin concentration and *URO-D*

Genotype		N	Iron, mg/g	Porphyrins, nmol/g	<i>URO-D</i> , nmol/h/mg
<i>URO-D</i>	<i>HFE</i>				
+/+	+/+	4	0.13 ± 0.04	0.75 ± 0.41	1.68 ± 0.09
+/+	+/-	5	0.15 ± 0.04	1.26 ± 0.56	1.53 ± 0.14
+/-	-/-	3	0.73 ± 0.14*	1.77 ± 1.42	1.36 ± 0.06
+/-	+/+	8	0.11 ± 0.02	8.46 ± 4.81	0.79 ± 0.07†
+/-	+/-	4	0.22 ± 0.05	2.13 ± 0.58	0.64 ± 0.07†
+/-	-/-	5	0.89 ± 0.22*	259.32 ± 43.15*†	0.22 ± 0.07*†

*Significantly different ($P < 0.05$) from *HFE*^{+/+} and identical *URO-D* genotype.

†Significantly different ($P < 0.05$) from *URO-D*^{+/+} and identical *HFE* genotype.

to 1 yr) were iron loaded by i.p. injection with iron-dextran (10 mg of iron) and were given drinking water supplemented with ALA (2 mg/ml) to bypass the rate-limiting step in hepatic porphyrin synthesis [ALA-synthase 1 (2, 3)]. Urinary porphyrin excretion before treatment was similar in animals of either genotype (Fig. 3). Animals were killed after 3 weeks of treatment when an increase in urinary porphyrin excretion was noted. In *URO-D*^{+/+} mice given iron and ALA, urinary porphyrin excretion rose 6-fold, and the predominant urinary porphyrin was coproporphyrin (Fig. 3). In contrast, urinary porphyrin excretion rose 29-fold in *URO-D*^{+/-} animals. Uroporphyrin and heptacarboxyl porphyrin predominated, but coproporphyrin excretion paralleled the increase seen in *URO-D*^{+/+} animals (Fig. 3).

Hepatic iron concentrations in animals injected with iron-dextran increased approximately 10-fold (Fig. 4A). There was no significant effect of ALA alone on porphyrin accumulation or *URO-D* activity in *URO-D*^{+/+} animals of either sex (Fig. 4B and C), but hepatic porphyrin accumulation (mainly coproporphyrin) increased slightly, and *URO-D* activity decreased slightly (Fig. 4B and C). This effect was more marked in females. In contrast, ALA or ALA plus iron caused a further reduction in *URO-D* activity in *URO-D*^{+/-} mice (Fig. 4B) without altering the levels of *URO-D* protein as determined by Western blotting (data not shown). Reduction of *URO-D* activity was accentuated by iron (Fig. 4B). Hepatic porphyrins accumulated in *URO-D*^{+/-} animals given ALA or ALA plus iron (Fig. 4C) and were predominantly uroporphyrin and heptacarboxylic porphyrin. Hepatic porphyrin accumulation was accentuated by iron in *URO-D*^{+/-} males but did not alter the high accumulation seen with ALA alone in females (Fig. 4C).

Genetic Hemochromatosis Produces a Porphyric Phenotype in *URO-D*^{+/-} Mice. Mice homozygous for a deletion of the *HFE* gene (*HFE*^{-/-}) develop hepatic iron overload by 10 weeks of age with liver iron concentrations approximately 8-fold higher than wt littermates (31, 34). Animals with the *HFE*^{+/-} genotype accumulate much less iron. We generated mice with the *URO-D*^{+/-}/*HFE*^{-/-} genotype on a C57BL/6J background through crossbreeding of the two mutant lines. Other genotypes resulting from these matings were *URO-D*^{+/+}/*HFE*^{+/-} and *URO-D*^{+/+}/*HFE*^{+/+}. Urine porphyrin excretion was monitored weekly beginning at 8 weeks of age. By 17 weeks of age, total urinary porphyrin excretion had increased 40-fold in *URO-D*^{+/-}/*HFE*^{-/-} mice with uroporphyrin and heptacarboxyl porphyrin predominating (data not shown). No change in urinary porphyrin excretion occurred in animals with other genotypes. Animals were killed between 14 and 17 weeks of age, and liver iron content, liver porphyrin content, and hepatic *URO-D* activities were measured. Hepatic porphyrins accumulated only in animals with the *URO-D*^{+/-}/*HFE*^{-/-} genotype, and in these animals, *URO-D* activity was reduced

to approximately 16% of control (*URO-D*^{+/+}/*HFE*^{-/-}) values (Table 1). The reduction in *URO-D* activity was more profound than observed in *URO-D*^{+/-} animals injected with iron-dextran (data not shown) even though hepatic iron levels were higher than in *URO-D*^{+/-}/*HFE*^{-/-} animals. Injected animals were killed 3 weeks after iron loading, whereas hepatic iron content in *URO-D*^{+/-}/*HFE*^{-/-} animals increased steadily from birth to the time of death, suggesting that the length of exposure to iron is important in generating the porphyric phenotype.

Discussion

The *URO-D*^{+/-} mouse, like most humans heterozygous for *URO-D* mutations, does not develop a porphyric phenotype unless additional factors are present, indicating that half-wt activity of *URO-D* does not create a rate-limiting step in the heme biosynthetic pathway. Supplementation of the drinking water with ALA to bypass the rate-limiting step caused an increase in urinary porphyrin excretion and hepatic accumulation in *URO-D*^{+/-} mice over a 3-week period. This effect was markedly accentuated by the injection of iron-dextran. *URO-D* activity fell well below the half-wt value, even though *URO-D* protein levels remained constant, suggesting that an inhibitor of *URO-D* is generated. Smith and Francis (35) reported that iron overload, produced by injection of iron-dextran, can cause hepatic porphyrin accumulation and diminished *URO-D* activity in mice with wt *URO-D* alleles, but these effects occurred 25 weeks after iron injection and varied widely between strains. Elder *et al.* (36) reported diminished *URO-D* activity, but no reduction in *URO-D* protein, in liver biopsy specimens from humans with sporadic-PCT, and iron depletion led to normalization of *URO-D* activity. Collectively, these data suggest that a common mechanism is responsible for expression of both F-PCT and sporadic-PCT, namely generation of an inhibitor of *URO-D* by an iron-dependent mechanism.

When *URO-D*^{+/-} animals were crossbred to *HFE*^{-/-} animals a porphyric phenotype appeared after 14 weeks without ALA supplementation. Thus, we have constructed a murine model of PCT in which no exogenous factors are required. We and others have reported that *HFE* mutations, hepatitis C virus infection, alcohol abuse, and estrogen use are factors contributing to the pathogenesis of PCT (1, 4, 5). In our study of 108 patients with PCT, multiple risk factors were identified in most cases (1). In three patients with F-PCT, however, homozygosity for the C282Y *HFE* mutation was the only risk factor identified. All three of these patients were children. Our murine model of F-PCT appears to reflect the clinical findings in humans. Unperturbed *URO-D*^{+/-} mice and *URO-D*^{+/-} humans appear normal, but the *URO-D*^{+/-}/*HFE*^{-/-} genotype is sufficient to generate the porphyric phenotype.

The importance of the genetic background on which iron overload occurs has been established in mice and is emphasized by the low incidence of PCT in humans with hemochromatosis. In a recent study of 184 hemochromatosis pedigrees, we detected PCT in 19 probands (10.3%; ref. 39). This value likely is higher than the true incidence of PCT in individuals with hemochromatosis, as some patients were ascertained for study because of signs and symptoms of PCT.

The *URO-D*^{+/-} genotype appears to both accelerate and accentuate the porphyric phenotype in the presence of iron overload. Our mouse model of F-PCT affords the opportunity to

define the iron-dependent mechanism leading to the generation of an inhibitor of URO-D activity without the confounding effects of exogenous perturbing factors.

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